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Bim is the key mediator of glucocorticoid-induced apoptosis and of its potentiation by rapamycin in human myeloma cells

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ABSTRACT

Glucocorticoids are widely used in anti-myeloma therapy and their action is potentiated by rapamycin, a mTOR inhibitor. However, the molecular mechanisms underlying these effects remain poorly characterized. We show here that dexamethasone (Dex)-induced apoptosis in MM.1S and OPM-2 cells is characterized by Bax and Bak conformational changes, $\Delta\Psi_m$ loss, cytochrome *c* release and caspase-3 activation. Rapamycin, which had minimal cytotoxic effect by itself, strongly potentiated Dex-induced apoptosis. Apoptotic gene expression profiling showed an increase in mRNA levels of Bim in MM.1S cells after Dex treatment and further increases in both cell lines when co-treated with rapamycin. Western blot analysis revealed a moderate increase in Bim protein levels in both MM.1S and OPM-2 cells. Immunoprecipitation experiments revealed that most Bim was complexed to Mcl-1 in untreated cells. Upon treatment with Dex, and specially Dex plus rapamycin, Bim–Mcl-1 complex was disrupted and Bim was found associated to a CHAPS-insoluble fraction. Overexpression of Mcl-1 stabilized Bim–Mcl-1 complexes upon treatment with Dex or Dex + rapamycin and fully prevented apoptosis. Gene silencing of Bim inhibited for the most part Dex-induced apoptosis and, to a large extent, apoptosis induced by Dex plus rapamycin. These results, taken together, indicate that Bim protein is the key mediator of apoptosis induced by Dex and also responsible for the potentiating effect of rapamycin, providing molecular criteria for the use of glucocorticoids combined with mTOR inhibitors in myeloma therapy.

1. Introduction

Glucocorticoid-containing regimes for the therapy of multiple myeloma (MM) were introduced more than 35 years ago [1] and still remain the most widely accepted treatment option in elderly patients [2]. From 1992, dexamethasone (Dex) alone is also frequently used in MM patients [3,4] and Dex has been considered to account for most of the responses to VAD (vincristine, adriamycin, dexamethasone) [4]. Although in 1995 it was found that the anti-myeloma action of Dex was due to apoptosis induction [5], the information concerning the molecular mechanism of glucocorticoid-induced apoptosis in MM cells is still scarce. Dex-induced apoptosis is independent of JNK activity [6] and takes place by the mitochondrial route, since it is inhibited by Bcl-2 overexpression [7] and occurs with down-regulation of Bcl-x_L mRNA levels [8,9]. Surprisingly it was reported that apoptosis induced by Dex involved the mitochondrial release of Smac/Diablo but not that of cytochrome *c* and, congruent with this, showed a poor caspase-3 activation [10,11].

It has also been reported that rapamycin, a specific inhibitor of mTOR activity, can sensitize MM cells to Dex-induced apoptosis [12]. A high-throughput microarray analysis revealed that expression of FasL, Bim and DR5 genes was up-regulated and that of XIAP and Bcl-2 genes down-regulated in OPM-2 cells by rapamycin [13]. Western blot analysis confirmed that rapamycin reduced levels of the caspase inhibitor XIAP [13]. However, the decrease of XIAP does not account *per se* for the potentiating effect of rapamycin on Dex-induced apoptosis. In this regard, we have recently found that rapamycin can increase Bim protein levels in some MM cell lines and thus potentiate apoptosis induced by interferon-α [14].

The aim of the present work was to define the key molecular determinants of Dex-induced apoptosis in myeloma cells as well as the mechanism underlying the apoptosis-potentiating effect of rapamycin. We have found that the BH3-only protein Bim is the key mediator of apoptosis induced by Dex and also responsible, for the most part, for the potentiating effect of rapamycin. These results provide molecular criteria to use Dex in single and combination chemotherapy and support the combined use of mTOR inhibitors with glucocorticoids in anti-myeloma therapy.

Abbreviations: Dex, dexamethasone; $\Delta\Psi_m$, mitochondrial membrane potential; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanine iodide; TMRE, tetramethylrhodamine ethyl ester; MM, multiple myeloma; PS, phosphatidylserine; RT-MLPA, reverse-transcriptase multiplex ligation-dependent probe amplification

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2. Experimental procedures

2.1. Cell culture, transfections and toxicity assays

Human multiple myeloma (MM) cell lines MM.1S and MM.1R, selected by its sensitivity and resistance, respectively, to Dex [8] were kindly provided by Dr. Atanasio Pandiella (CIC, Salamanca). U266 and RPMI 8226 were from ATCC and OPM-2 from DSMZ (Germany). Cells were routinely cultured at 37 °C in RPMI 1640 medium supplemented with 10% or 15% (OPM-2) fetal calf serum (FCS), L-glutamine and penicillin/streptomycin (hereafter, complete medium). Mcl-1 stable-overexpressing MM.1S cells were generated as previously described [14]. In brief, the pLZR-IRES-GFP-Mcl-1 plasmid, encoding the full-length human Mcl-1 cDNA (kindly provided by Dr. Xiandong Wang, Univ. of Texas, USA), was transduced by retroviral infection. Clones derived from single cells were obtained by limiting dilution and the expression of Mcl-1 was analyzed by Western blotting. Cells containing empty vector (pLZR-IRES-GFP) were used as controls. In proliferation assays, cells (3×10^5 cells/ml) were treated in flat-bottom, 24-well (1 ml/well) or 96-well plates (100 ml/well) with 5 μM dexamethasone (Dex) in complete medium for the times indicated. When appropriate, the pan-caspase inhibitor Z-VAD.fmk (100 μM, Bachem, Germany), and the mTOR inhibitor rapamycin (10 nM, Calbiochem) were added to cells 1 h prior to Dex treatment. Cell proliferation was determined by a modification of the MTT-reduction method [15] and viability by microscopical inspection of Trypan blue-stained cells. Nuclear alterations during apoptosis were analyzed by fluorescence microscopy in cell cultures labeled with Hoechst 33342 (Invitrogen, Barcelona, Spain).

2.2. RT-MLPA

Cellular mRNA was analyzed by reverse transcriptase multiplex ligation-dependent probe amplification (RT-MLPA), using SALSA MLPA KIT R011 Apoptosis mRNA from MRC-Holland (Amsterdam, The Netherlands) [16]. Total RNA from 5×10^6 cells was isolated from myeloma cells by the RNeasy Mini Kit (Qiagen, Barcelona, Spain) according to the manufacturer's protocol. Sample homogenization was improved by passing the lysed cellular sample obtained with the RLT buffer through a QiaShredder column (Qiagen). To perform RT-MLPA analysis 200 ng of total RNA were first reverse-transcribed using the gene-specific probe mix. The resulting cDNA was annealed overnight at 60 °C to the MLPA probe mix, and then ligated. Ligation products were amplified by PCR (35 cycles, 30 s at 95 °C, 30 s at 60 °C and 1 min at 72 °C) with one unlabelled and one FAM-labeled primer. The final PCR amplified fragments were separated by capillary electrophoresis on a 48-capillary ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Madrid, Spain). The peak area and height were processed using PeakScanner analysis software. The levels of mRNA for each gene were expressed as a normalized ratio of the peak area divided by the peak area of housekeeping control gene (β_2-microglobulin). This accounted for the relative abundance of mRNAs of apoptotic genes. The kit contains probes for mRNAs of 39 apoptosis-related genes. In the R011 mix, the probe signals of, Bcl-x_L, Apollon/BRUCE, Bnip3L and Mcl-1 mRNAs are deliberately reduced by the inclusion of competitor oligonucleotides.

2.3. RNA interference assays

MM.1S cells were electroporated with siRNA oligonucleotides by using a Nucleofector system (Amaxa, Germany) according to the manufacturer's instructions. Briefly, 3×10^6 cells were resuspended in 100 μl solution V containing 90 pmol double-stranded siRNAs and electroporated with O23 program. Prewarmed cultured medium (500 μl) was added to each cuvette and cells were transferred to plastic dishes at 6×10^5 cells/ml. OPM-2 cells were transfected with HiPerFect Transfection Reagent (Qiagen). Briefly, one day prior transfection, cells were diluted to a density of 3×10^5 cells/ml in complete medium. The next day, 4×10^5 cells/well in a 24-well plate were resuspended in 100 μl of complete medium. The corresponding siRNA (30 pmol/10^6 cells) and HiPerFect (2.5 μl/well) were added to an Eppendorf tube containing 100 μl of medium without serum per well and incubated for 10 min at room temperature. The complexes were added drop-wise onto the cells, mixed gently and incubated at 37 °C for 6 h. Then, complete medium (up to 1 ml) was added to each well. Transfected cells were cultured for 6 or 36 h, as indicated, before Dex and/or rapamycin addition and further incubated for another 24 h.

The sense strand sequences used, with two 3′-dTdT overhangs, were as follows:

Bim-siRNA: 5′-GACCGAGAAGGUAGACAAUUG-3′
Puma-siRNA1 (p1): siGENOME SMARTpool
Puma-siRNA2 (p2): 5′-GCCTGTAAGATACTGTATA-3′
Noxa-siRNA: 5′-CUUCCGGCAGAAACUUCUG-3′
Bmf-siRNA: 5′-CAAGGTGTCATGCTGCCTT-3′
Lamin A/C-siRNA: 5′-CTGGACTTCCAGAAGAACA-3′ (negative control).

Bim, Noxa, Lamin A/C and Puma (p1) oligonucleotides were synthesized by Dharmacon (Madrid, Spain) and Puma (p2) and Bmf oligonucleotides were from Qiagen (Spain).

2.4. Flow cytometry analysis

Intracellular levels of FasL and activation of caspase-3 were determined by immunolabeling with specific antibodies. After the different treatments, cells (1×10^6) were fixed with 4% paraformaldehyde in PBS (15 min, 4 °C), permeabilized with 0.1% saponin in PBS containing 5% goat serum for 15 min and incubated for 30 min at 20 °C with either mouse anti-human FasL mAb (NOK-1) and rabbit anti-mouse IgG-FITC or anti active caspase 3-FITC mAb (C92-605), both from BD Biosciences (Madrid, Spain). Cells were resuspended in 500 μl PBS and analyzed in a flow cytometer (FACScan). Apoptosis in MM.1S cells was evaluated by the simultaneous determination of phosphatidylserine (PS) exposure and mitochondrial membrane potential ($\Delta\Psi_m$) in the same cell sample. Briefly, cells (2.5×10^5 in 200 μl) were incubated with 2 nM 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6(3)$, Invitrogen) at 37 °C for 10 min in annexin-binding buffer (140 mM NaCl, 2.5 mM $CaCl_2$, 10 mM Hepes/NaOH, pH 7.4). Then, 0.4 μl annexin V-PE (Immunostep, Salamanca, Spain) were added and incubated at room temperature for 15 min. Cell suspension was diluted to 500 μl with binding buffer and analyzed by flow cytometry. In OPM-2 cells, displaying non-specific binding of the PE moiety of annexin V-PE, $\Delta\Psi_m$ was determined with tetramethylrhodamine ethyl ester (TMRE, 60 nM) and PS exposure with annexin V-FITC (1 μl), all from Invitrogen.

Conformational changes of Bax and Bak proteins were assessed by intracellular immunostaining using specific antibodies recognizing only the pro-apoptotic conformation of these proteins [17]. Cells (1×10^6) were cultured in the presence or absence or 5 μM Dex, 10 nM rapamycin or both for 24 h. Then, cells were fixed with 1% paraformaldehyde in PBS (15 min, 4 °C) and incubated for 25 min at room temperature with 0.5 μg of anti-Bax (6A7, BD Biosciences) or anti-Bak (TC100, Calbiochem) in 100 μl of PBS containing 0.1% saponin and 5% goat serum. Cells were washed with 0.03% saponin in PBS, incubated with a FITC-labeled anti-mouse IgG antibody (Invitrogen) and analyzed by flow cytometry. Quantitative analysis of cytochrome *c* release from mitochondria during apoptosis was assayed by the method of Waterhouse and Trapani [18]. In brief, cells (1×10^6) were treated with 5 μM Dex in the presence or absence of rapamycin, permeabilized with 100 μl digitonin (25–50 μg/ml in PBS containing 100 mM KCl) for 5 min on ice, fixed in 4% paraformaldehyde in PBS and resuspended in blocking buffer (3% BSA, 0.05% saponin in PBS). Cells were incubated overnight at 4 °C with a 1/200 dilution of anti-native cytochrome c mAb (6H2.B4, BD Biosciences) in blocking buffer, washed, incubated with a FITC-labeled secondary antibody and analyzed by flow cytometry.

2.5. Immunoprecipitation assays

Total protein extracts from control or treated cells (3×10^6) were prepared by suspension in 400 μl CHAPS buffer (1 mM HEPES pH 7.4, 150 mM NaCl, 2% CHAPS). Cell lysates were centrifuged (13,000×*g*, 10 min) and supernatants subjected to immunoprecipitation with 3 μg of either mouse mAb anti-Mcl-1 (clone 22, BD biosciences) or rat mAb anti-Bim (clone 14A8, Calbiochem), at 4 °C, overnight, with gentle rotation. Protein–antibody complexes were recovered by incubation with anti-mouse or anti-rat agarose-IgG beads (Sigma) at 4 °C for 3 h and beads recovered by brief centrifugation. Beads containing immunoprecipitates were washed three times with CHAPS buffer and proteins released by heating for 5 min at 100 °C. Proteins from immunoprecipitates, supernatants and pellets were analyzed by SDS-PAGE and Western blotting using rabbit primary antibodies to avoid detection of mouse or rat immunoglobulins. Anti-cytochrome *c* oxidase, subunit I, mAb (clone 1D6E1A8, Invitrogen) was used to localize mitochondria-rich fraction.

2.6. Western blot analysis

Levels of proteins involved in apoptosis were analyzed by Western blotting. In general, membrane filters were sequentially analyzed for several proteins by a multiple blotting assay method, as described [19]. After the corresponding treatments, cells were lysed in 50 mM Tris/HCl pH 7.4 buffer containing 0.15 mM NaCl, 10% glycerol, 1 mM Na_3VO_4, 10 mM $Na_4P_2O_7$, 50 mM NaF, 1 mM EDTA, 10 mg/ml leupeptin, 1 mM PMSF and 1% Triton X-100. Solubilized proteins from equal numbers of Trypan-blue negative cells (3×10^5/lane) were resolved by SDS-15% PAGE, transferred to PVDF membranes and incubated with primary antibodies diluted in TBS-T (10 mM Tris/HCl pH 8.0, 0.12 M NaCl, 0.1% Tween-20), containing 5% skimmed milk. Primary antibodies anti human proteins used were: anti-XIAP (clone 48) and anti-Bax (#554104) both from BD Biosciences; anti-survivin (#NB500-201, Novus); anti-Bim (22-40, Calbiochem), anti-Bcl-2 (sc-783) and anti-Mcl-1 (sc-819) from Santa Cruz Biotechnology; anti-Bak (#06-536, Millipore); anti-PUMA (#4976), anti-Bmf (#4692) from Cell Signalling and anti-Noxa (clone 114C307, Abcam). Membranes were washed with TBS-T and incubated with 0.2 μg/ml of the corresponding peroxidase-labeled secondary antibody (Sigma) and revealed by ECL. Control of protein loading was achieved by reprobing with anti-actin (1/10,000) or anti-tubulin (1/10,000) (Sigma). When necessary, the intensity of bands was quantified by densitometry by using Quantity One software (BioRad). The intensity of each Bim isoform was normalized relative to that of the corresponding actin band.

2.7. Statistical analysis

Analysis of significance of results was evaluated, when necessary, by two-tailed Student *t* test using GraphPad Prism 4.0 software (GraphPad

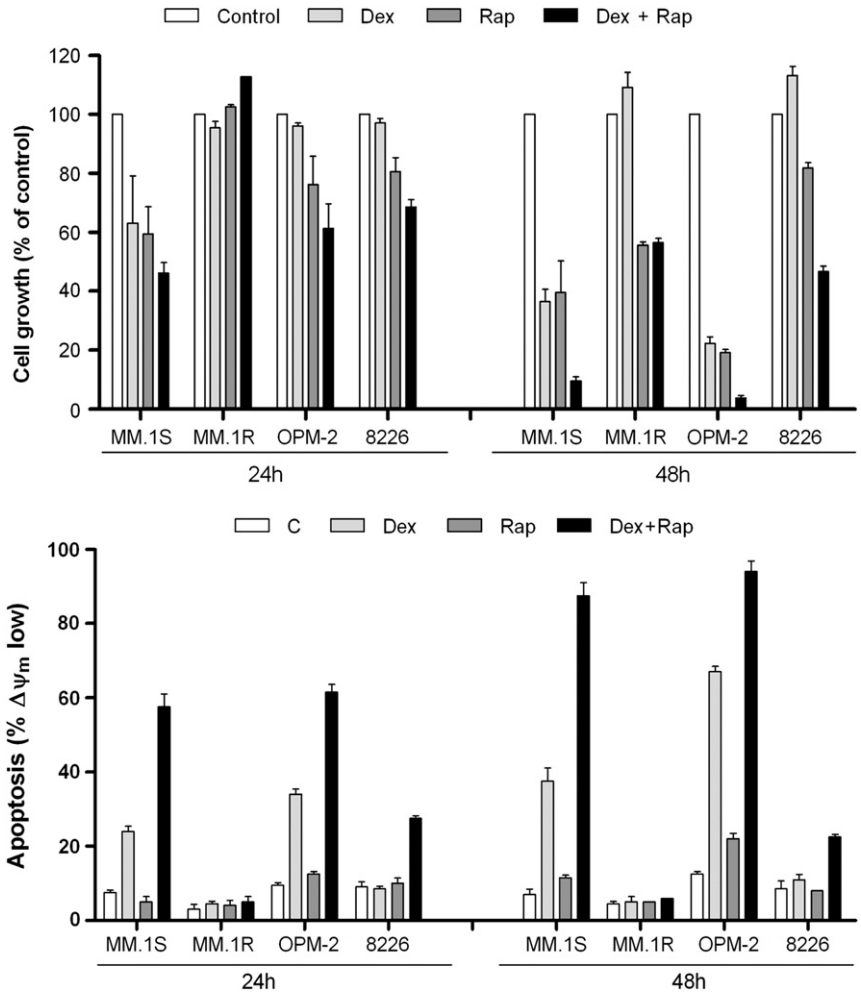

Fig. 1. Effect of dexamethasone and rapamycin on cell growth (top) and apoptosis (bottom) in MM cells. MM.1S, MM.1R, OPM-2 and RPMI 8226 cells (300,000 cell/ml) were treated with dexamethasone (Dex, 5 μM), rapamycin (Rap, 10 nM) or both, as indicated. After 24 or 48 h of culture, cell viability was determined by the MTT-reduction method and apoptosis by measuring mitochondrial membrane potential ($\Delta\Psi_m$) with $DiOC_6(3)$ by flow cytometry. Results are the mean ± SD, of three to five independent determinations per point.

Software, Inc., San Diego, CA, USA). Values of p between $0.01<p<0.05$ were considered to be significant (*), very significant (**) when $0.001<p<0.01$, and extremely significant (***) when $p<0.001$.

3. Results

3.1. Effect of Dex and rapamycin on proliferation of MM cells

Treatment with 5 μM Dex for 24–48 h caused a time-dependent growth inhibition in sensitive MM.1S and OPM-2 cells (Fig. 1), which was mainly due to the induction of apoptosis, characterized by loss of $\Delta\Psi_m$ (Fig. 1) and PS exposure (not shown). The apoptotic effect of Dex increased with time (Fig. 1). By contrast, Dex treatment had no significant effect on growth and apoptosis induction in MM.1R and RPMI 8226 cells (Fig. 1), according to published data [12]. The mTOR inhibitor rapamycin inhibited growth of MM.1S and OPM-2 cells by about 60% and 80%, respectively. after 48 h treatment, causing an increase in the proportion of cells in the G_1 phase of cell cycle (not shown). Except for the PTEN null OPM-2 cells, which are stringently dependent on the PI3K/Akt for survival [20,21], rapamycin, at the doses used, did not induce a significant apoptosis in any cell line (Fig. 1). However, the combination of Dex with rapamycin strongly potentiated Dex-induced apoptosis in MM.1S and OPM-2 cell lines, slightly increased the growth inhibitory effect of rapamycin in RPMI 8226 cells and had no significant effect on MM.1R cells (Fig. 1). U266 cells, as reported previously [12], were resistant to treatment with Dex alone or in combination with rapamycin (data not shown).

3.2. Characteristics of apoptosis induced by Dex and Dex plus rapamycin

Dex treatment induced proapoptotic conformational changes of Bax and Bak, which were increased by combination with rapamycin (Fig. 2a). Dex also induced the release of appreciable amounts of cytochrome *c* from mitochondria, as determined by flow cytometry, and this release was increased by rapamycin co-treatment (Fig. 2b). Apoptosis was also characterized by a significant caspase-3 activation, which was fully prevented by co-treatment with the pan-caspase inhibitor Z-VAD.fmk (Fig. 2c). Z-VAD.fmk also prevented the development of classical apoptotic morphology (not shown) but it only inhibited in part Dex-induced $\Delta\Psi_m$ loss and cell death (from 50% in the absence to 42% in the presence of Z-VAD.fmk in MM.1S cells). When combined with Dex, rapamycin strongly potentiated $\Delta\Psi_m$ loss, PS exposure, Bax and Bak conformational changes, cytochrome *c* release, caspase-3 activation, and apoptosis (Figs. 1 and 2). Co-treatment with Z-VAD.fmk prevented again caspase-3 activation (Fig. 2c) but only

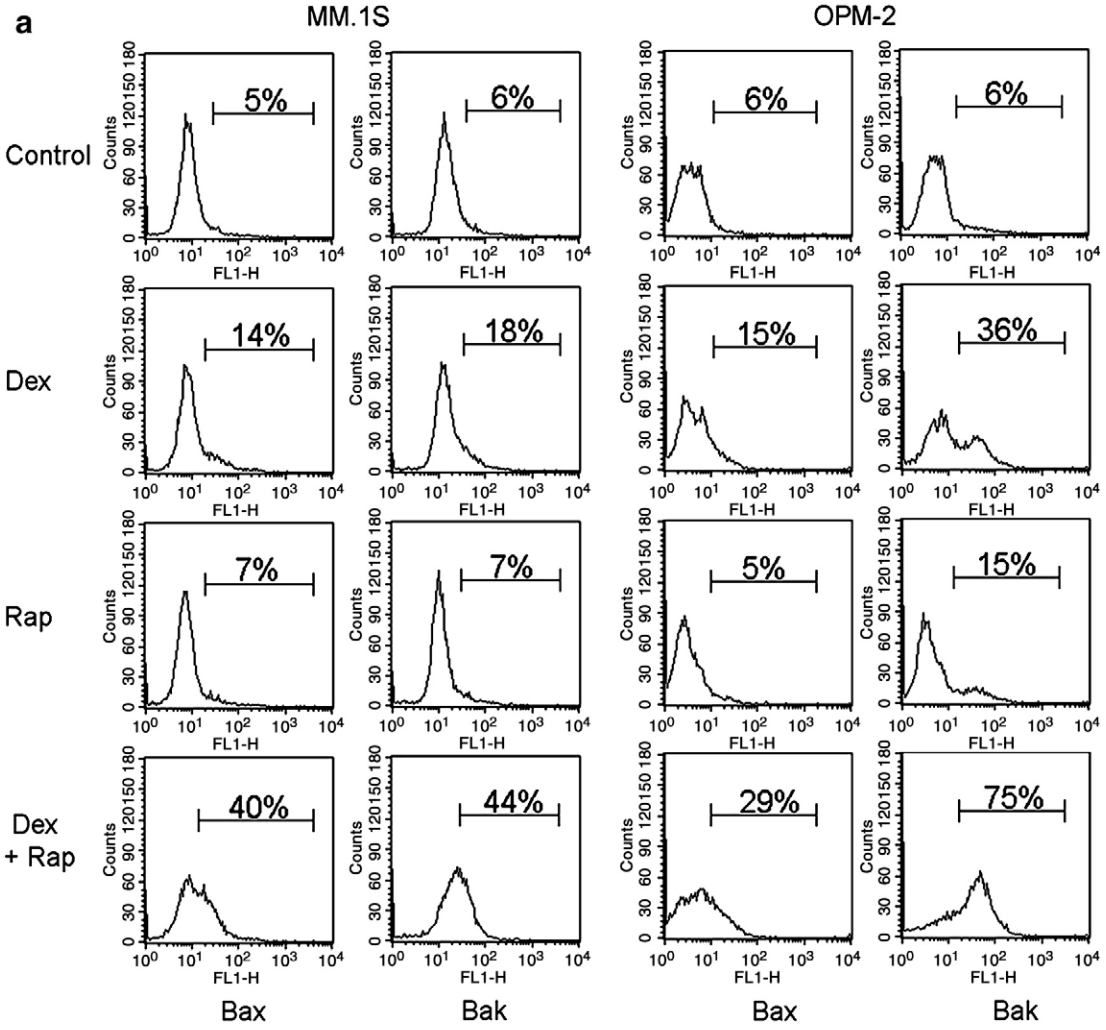

Fig. 2. Characteristics of Dex and rapamycin induced apoptosis. MM.1S and OPM-2 cells (300,000 cell/ml) were treated with either 5 μM Dex, 10 nM rapamycin (Rap) or both, for 24 h and analysis of (a) proapoptotic conformational changes of Bax and Bak was performed by flow cytometry with specific antibodies, as described in Experimental procedures. (b) Cytochrome *c* release from mitochondria and (c) caspase-3 activation were analyzed by flow cytometry, with specific antibodies, in MM1.S and OPM-2 cell lines treated with either Dex, Rap or both for 40 h, as described in Experimental procedures. Specificity of caspase-3 activation was assessed by co-incubation with the pan-caspase inhibitor Z-VAD.fmk (100 μM).

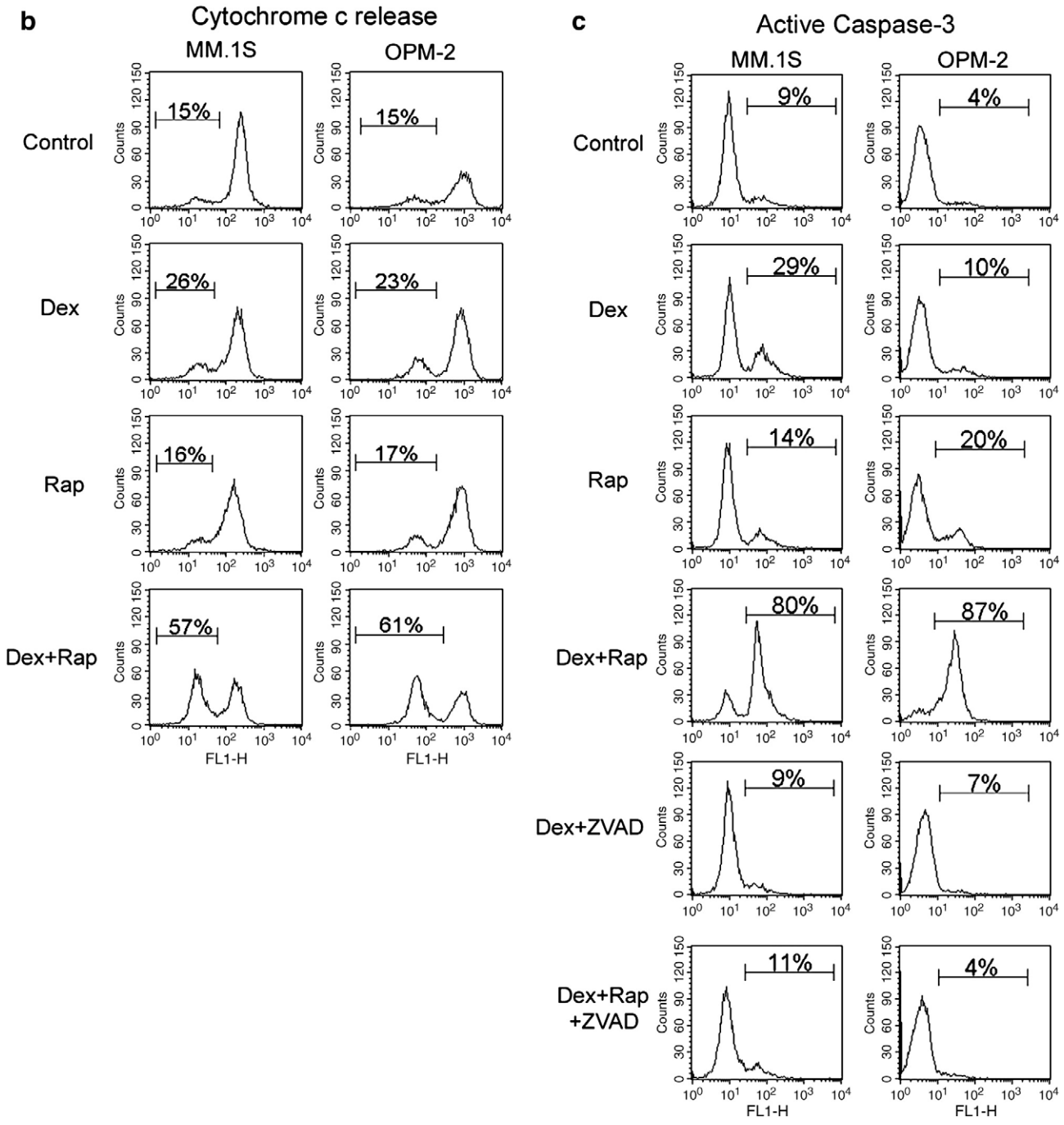

Fig. 2 (*continued*).

partly inhibited cell death (from 94% in the absence to 80% in the presence of Z-VAD.fmk in MM.1S cells). Z-VAD.fmk did not prevent either Bax and Bak conformational changes or cytochrome *c* release (data not shown).

Rapamycin alone had minimal effects on $\Delta\Psi_m$ loss, PS exposure, Bax and Bak conformational changes, cytochrome *c* release and caspase-3 activation. On the other hand, the Fas/FasL system was not involved in Dex-induced apoptosis. Flow cytometry analysis revealed that neither OPM-2 nor MM.1S cells expressed FasL in plasma membrane, though around 85% of OPM-2 and 95% of MM.1S cells expressed intracellular FasL (Fig. S1). FasL levels were not significantly altered by treatment with Dex, rapamycin or both and blocking anti-Fas antibodies did not prevent apoptosis induced by Dex and Dex plus rapamycin (not shown).

3.3. Effect of Dex and rapamycin in mRNA levels of apoptotic proteins

We used the RT-MLPA technique to investigate changes in expression levels of 39 genes involved in apoptosis, mainly in the intrinsic pathway, during treatment with Dex and rapamycin. In MM.1S cells, Dex moderately increased the mRNA levels of the BH3-only proteins Bim (1.6-fold) and to a lesser extent those of Puma and Bmf, as well as those of the anti-apoptotic protein Mcl-1 (Fig. 3). Treatment with rapamycin alone for 24 h slightly increased mRNA levels of Puma, Bcl-2 and Mcl-1. Combined treatment with Dex and rapamycin further increased the mRNA levels of Bim (3.8-fold), Puma, Bcl-2 and Mcl-1. Noxa mRNA levels were low and decreased upon treatment. These reductions in Noxa mRNA levels were also found in Dex-insensitive MM.1R cells and in low sensitive

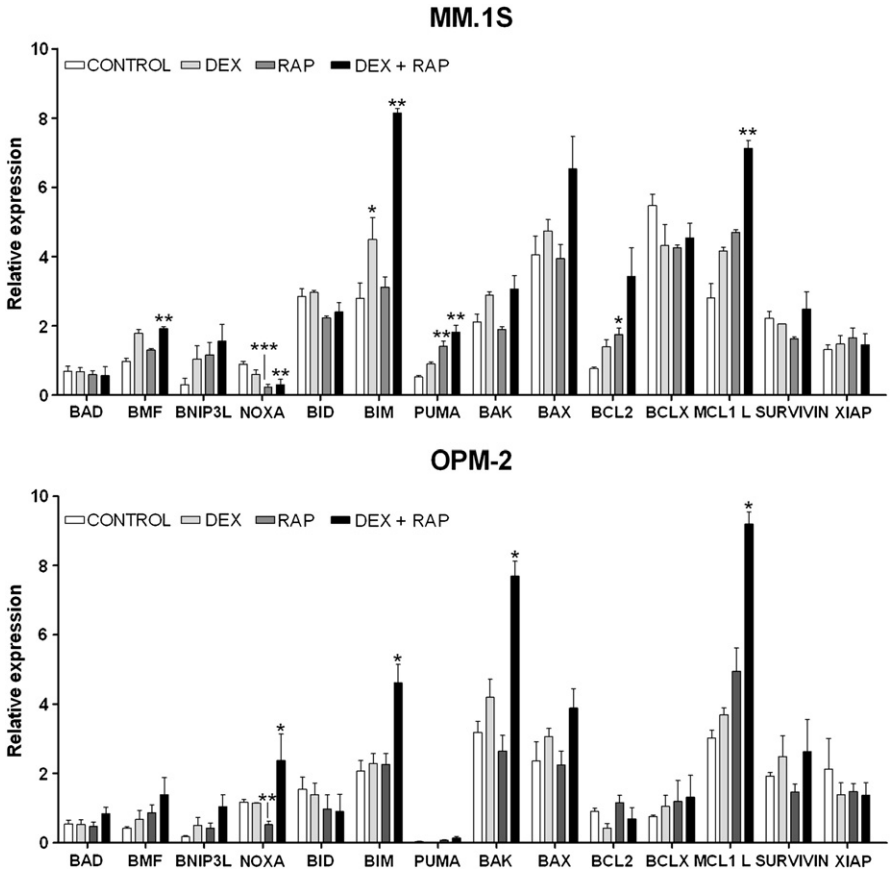

Fig. 3. Apoptotic gene expression analysis by RT-MLPA. MM.1S and OPM-2 cells (300,000 cell/ml) remained untreated (white bars) or were treated with either 5 μM Dex, 10 nM rapamycin (Rap) or both for the times indicated. Total RNA from of 5×10^6 cells was extracted as described in Experimental Procedures and 200 ng were used for RT-MLPA analysis. The mRNA amount of all genes was normalized with respect to that of β_2-microglobulin. Results are expressed as the mean ± SEM of the relative mRNA expression levels from three individual determinations in three different experiments. Statistical significant differences of treated versus untreated cells: *$p<0.05$; **$p<0.01$ and ***$p<0.001$.

RPMI 8226 cells (Fig. S2). No significant changes in Bim and Mcl-1 mRNA levels were found in MM.1R and RPMI 8226 cells treated with either Dex or Dex + rapamycin (Fig. S2). RT-MLPA analysis of OPM-2 cells showed a slight increase in transcriptional activity of the Bim gene after 24 h treatment with Dex, which was greatly increased by co-treatment with rapamycin (Fig. 3). The combination of Dex and rapamycin also increased Noxa mRNA levels in this cell line. Puma mRNA levels remained very low in any experimental condition and, as found in MM.1S cells, those of Mcl-1 increased (Fig. 3).

3.4. Effect of Dex and rapamycin on levels of apoptotic proteins

Changes in levels of relevant apoptotic proteins were analyzed by Western blot. In MM.1S cells, treatment with Dex increased Bim_L levels and the combination of Dex + rapamycin increased those of Bim_L and Bim_S nearly two-fold (Fig. 4). Mcl-1 levels also slightly increased after Dex or rapamycin treatment and were reduced by Dex and rapamycin combination (Fig. 4), according to the high caspase-3 activation observed (Fig. 2c). Dex and rapamycin did not significantly change levels of XIAP and survivin (Fig. 4) but combined treatment with Dex and rapamycin greatly reduced levels of these proteins. Levels of Puma-β increased slightly after 24 h treatment with either Dex or rapamycin but combined treatment with Dex and rapamycin led to an abrupt decrease in levels of this protein. MM.1S cells expressed very low levels of Noxa and they did not increase upon treatment. In OPM-2 cells, levels of all Bim isoforms increased by 24 h of incubation with Dex. Combined treatment with Dex and rapamycin increased levels of Bim_L and Bim_S isoforms, although to a lesser extent than those observed in MM.1S cells (Fig. 4). Levels of Mcl-1 did not significantly change after treatment with either Dex or rapamycin and were slightly reduced by the combined treatment (Fig. 4). Noxa was significantly expressed by OPM-2 cells under basal conditions and their levels decreased during treatment with Dex or rapamycin (Fig. 4). Levels of XIAP and survivin decreased in cells treated with Dex and rapamycin (Fig. 4) coinciding in time with the increase in caspase-3 activation and apoptosis (Fig. 2c). No significant changes in Bim protein levels were found in MM.1R and RPMI 8226 cells treated with Dex or Dex + rapamycin (Fig. S3).

3.5. Interaction between Bim and Mcl-1 during Dex-induced apoptosis

Since it has been reported that Mcl-1 acts as a natural antagonist of Bim in myeloma and leukemia cells [22,23] we analyzed the effect of Mcl-1 overexpression on Dex-induced cell death. Apoptosis induced by Dex in the presence or absence of rapamycin, after 24 or 48 h treatment, was fully prevented by overexpression of the anti-apoptotic protein Mcl-1 (Fig. 5). To gain insight into the mechanism of apoptosis induction by Bim and its blocking by Mcl-1, co-immunoprecipitation experiments were performed (Fig. 6). When Mcl-1 was immunoprecipitated, most Bim was associated to Mcl-1 in untreated MM.1S cells (Fig. 6a). Low amounts of Bim were found in the supernatant and CHAPS-insoluble membrane pellet. When MM.1S cells were treated with Dex and immunoprecipitated with anti-Mcl-1, the amount of Bim in IP supernatants and that associated to membrane insoluble fraction, slightly increased (Fig. 6a). Treatment with rapamycin alone gave a distribution pattern similar to that of

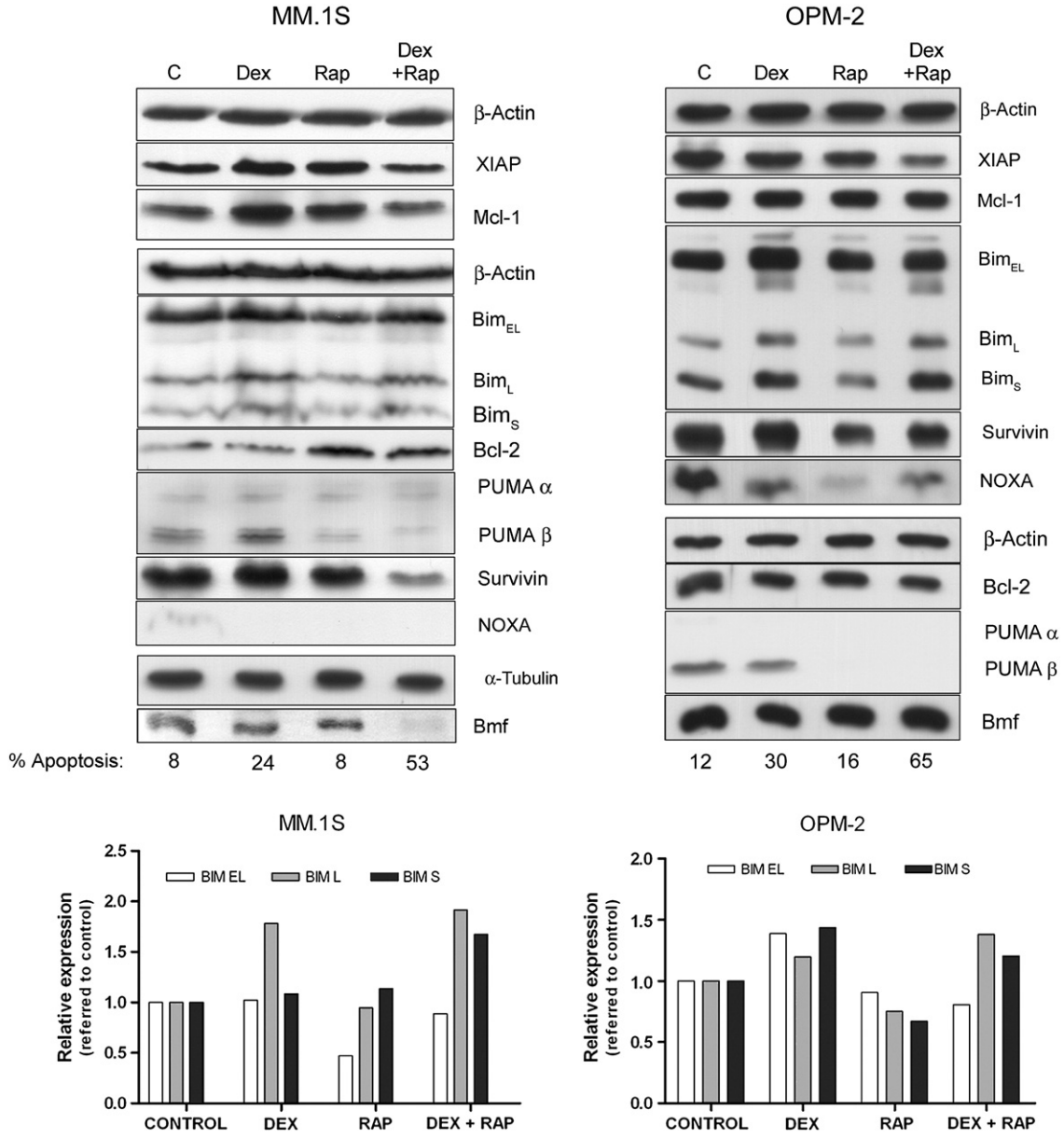

Fig. 4. Levels of Bcl-2 family and other apoptotic proteins in cells treated with Dex and/or rapamycin. MM.1S and OPM-2 cells were treated with either 5 μM Dex, 10 nM rapamycin or both, for 24 h. Cells (3×10^5/lane) were lysed in 1% Triton X-100 buffer and levels of relevant apoptotic proteins analyzed by SDS-PAGE and Western blotting with specific antibodies. Actin or tubulin were used as loading markers and are shown at the top of each blotting membrane analyzed, as indicated. The percentage of cell death, determined by Trypan-blue staining, is indicated in each case. Densitometric analysis of Bim levels (normalized to those of actin), corresponding to the blots shown, was performed by using Quantity One software.

control cells. In MM.1S cells treated with Dex and rapamycin, in which the total amount of Mcl-1 in Triton X-100 cell lysates decreased (Fig. 4), the relative amount of Bim bound to Mcl-1 decreased and a significant amount of Bim was recovered in the CHAPS-insoluble membrane pellet (Fig. 6b). Similar results, concerning the association of Bim with Mcl-1 and the association of Bim with CHAPS-insoluble membrane pellet, were obtained when Bim was immunoprecipitated (Fig. 6b). No significant interaction of Bim with Bak, Bax (Fig. 6b) or Bcl-x_L (data not shown) could be noticed by immunoprecipitation in cells either untreated or treated with Dex. Immunoprecipitation of Bim in MM.1S cells overexpressing Mcl-1 (MM.1S/Mcl-1) revealed that this protein remained bound to Mcl-1 in untreated cells and cells treated with Dex or with Dex + rapamycin, in agreement with the lack of apoptosis observed. No association of Bim to membrane pellet was found in cells overexpressing Mcl-1 under any experimental condition (Fig. 6c).

3.6. Effect of gene silencing of BH3-only proteins on Dex-induced apoptosis

To further precise the role of BH3-only proteins in Dex-induced apoptosis, we transfected myeloma cells with siRNAs for Bim, Puma, Noxa and Bmf. Down-regulation of Bim protein levels in MM.1S cells by transfection with Bim-siRNA (Fig. 7a) prevented virtually all of Dex-induced toxicity and reduced apoptosis induced by Dex and rapamycin by about 50% (Fig. 7b). Unexpectedly, Puma-siRNA1 probe mix caused increases in basal Bim levels (Fig. S4a) and increased Dex-induced apoptosis (Fig. S4b) at 24 h. A comparable effect was previously reported with a similar probe Puma-siRNA mix [24]. Transfection with Puma-siRNA2 caused a slight, but significant ($p<0.05$) protective effect in cells treated with Dex or Dex plus rapamycin, but it slightly decreased Bim levels (Fig. 7a). Combined transfection with Bim-siRNA and Puma-siRNA2 did not significantly

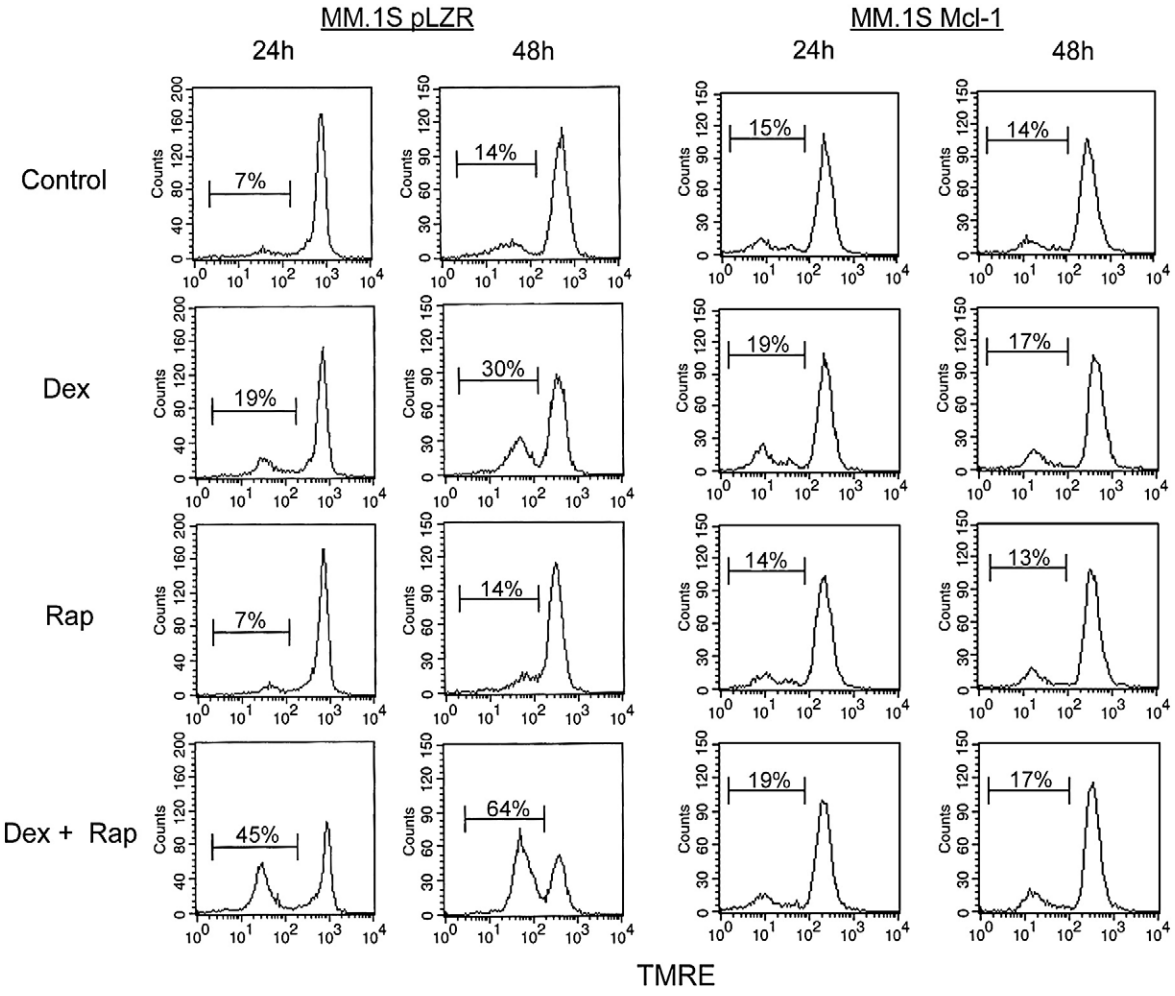

Fig. 5. Effect of Mcl-1 overexpression on Dex-induced apoptosis. MM.1S/pLZR and MM.1S/Mcl-1 cells were treated for 24 or 48 h with either 5 μM Dex, 10 nM rapamycin or both, as indicated, and apoptosis evaluated by determining $\Delta\Psi_m$ with TMRE by FACS analysis. A single representative experiment, out of three of each experimental condition, is shown.

improved protection offered by Bim-siRNA alone in apoptosis induced with Dex and rapamycin (data not shown). Western blot analysis confirmed the reduction in levels of Bim and Puma-α in cells transfected with the corresponding siRNAs at the indicated time (Fig. 7a). Gene silencing with Bmf-siRNA had no significant effect on apoptosis induced by Dex alone or in combination with rapamycin (Fig. S5).

In OPM-2 cells, transfection with Bim siRNA also abrogated most of the toxicity induced by Dex alone ($p<0.05$) and around 50% of apoptosis caused by Dex in combination with rapamycin. Bim gene silencing efficiently reduced Bim levels (mainly Bim_L and Bim_S) during treatment with Dex, rapamycin or both (Fig. 7c). Since OPM-2 cells expressed significant Noxa levels (Fig. 4) and treatment with Dex and rapamycin increased the relative amount of Noxa mRNA (Fig. 3) we also analyzed the effect of Noxa gene silencing in apoptosis. Noxa siRNA treatment reduced Noxa basal levels but it did not prevent apoptosis induced with Dex plus rapamycin (Fig. 7b, c).

4. Discussion

Glucocorticoids have been used for more than 35 years in MM therapy and in particular in elderly patients [2]. Dexamethasone, alone or combined with thalidomide, lenalidomide or bortezomib, is used in patients subjected to high-dose therapy [25]. Recently it has been reported that rapamycin, a specific inhibitor of mTOR, sensitizes MM cells to Dex-induced apoptosis [12,13]. However, the lack of a molecular explanation for this sensitization and even of the detailed molecular mechanism of Dex-induced apoptosis in MM cells, seriously hamper the use of this combination in therapy.

It was reported that Dex-induced apoptosis in MM cells was inhibited by Bcl-2 overexpression [7] and entailed the mitochondrial release of Smac/Diablo, but not that of cytochrome *c*, leading to a poor caspase-3 activation [10,11]. Experiments using oligonucleotide arrays have indicated that Dex treatment down-regulated Bcl-x_L mRNA levels in MM cells [8,9]. However, there is no information available concerning what proteins are the key inducers of apoptosis. Our current results indicate that Dex-induced apoptosis fulfills the molecular characteristics of the intrinsic pathway of apoptosis, including Bax and Bak conformational changes, loss of $\Delta\Psi_m$, and PS exposure. Moreover, by using sensitive flow cytometric assays, we have detected the mitochondrial release of cytochrome c and caspase-3 activation after Dex treatment. We also confirm the previously reported reduction in Bcl-x_L mRNA levels in MM.1S cells treated with Dex, though levels of Bcl-x_L protein remained roughly constant. More important, we have found a significant increase both at the mRNA and to a lesser extent, protein level, of the BH3-only protein Bim [26] during apoptosis. This suggested that Bim could be the responsible for triggering the proapoptotic conformational changes of Bax and Bak and the release of apoptotic mediators from mitochondria. The fact that more Bak than Bax underwent Dex-induced conformational changes in OPM-2 cells suggests that the role of Bak is more important in the induction of apoptosis in this particular cell line. A preferential implication of Bak over Bax has been previously found in other process of Bim-induced apoptosis [14]. Immunoprecipitation and

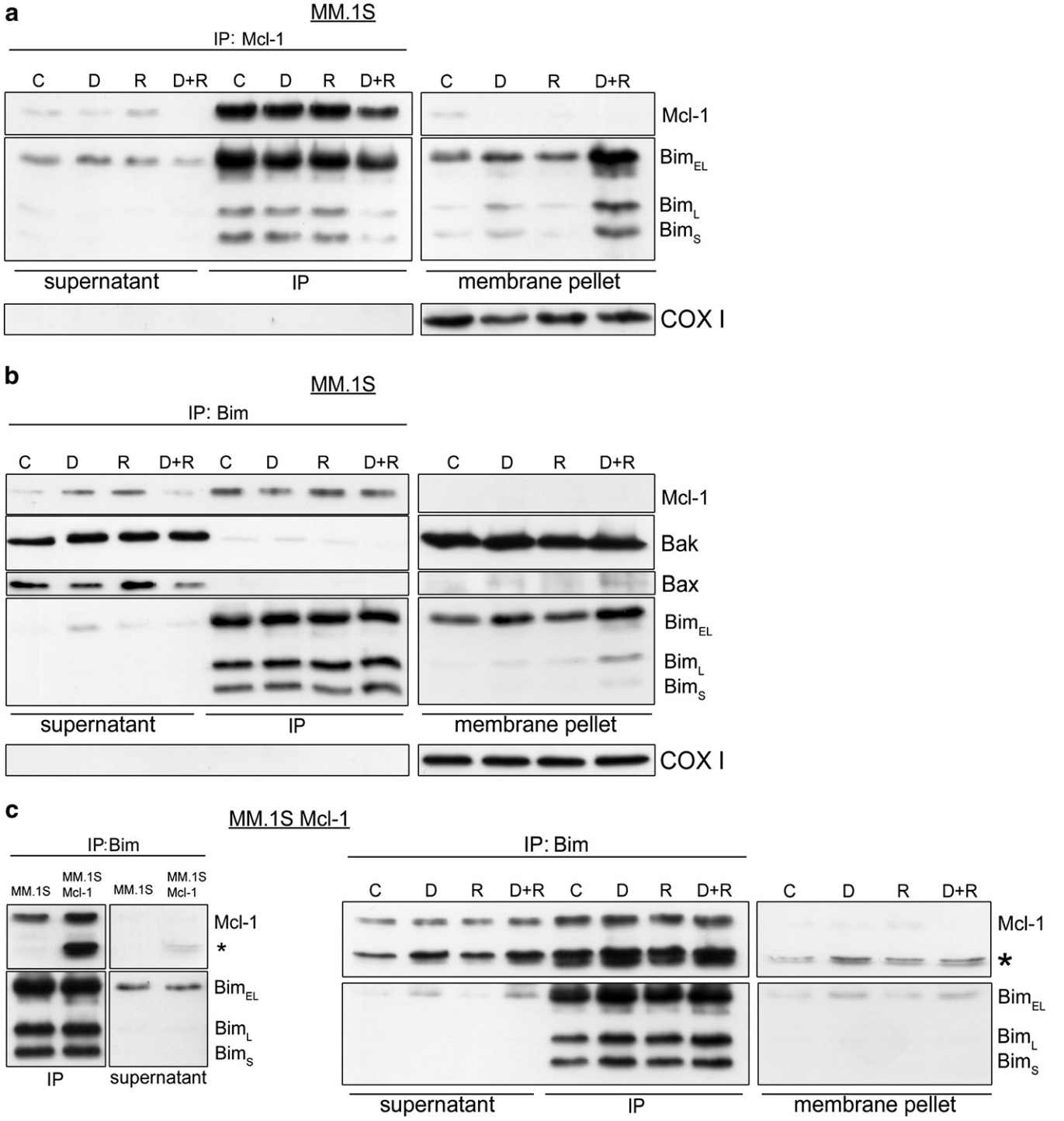

Fig. 6. Evaluation of Bim-Mcl-1 interaction during apoptosis. MM.1S cells (5×10^6 per point) were left untreated (C) or incubated for 24 h with 5 μM Dex (D), 10 nM rapamycin (R) or both (D + R), homogenized in a 2% CHAPS-containing buffer and subjected to immunoprecipitation with (a) anti-Mcl-1 (clone 22) or (b) anti-Bim (clone 14A8) antibodies, as indicated. Insoluble membrane pellets, immunoprecipitation supernatants and immunoprecipitates (IP) were analyzed by SDS-PAGE and Western blot and revealed with anti-Bim, anti-Mcl-1, anti-Bak and anti-Bax antibodies. Mitochondrial membrane fraction was identified with aid of an anti-cytochrome c oxidase, subunit I (COX I), mAb. (c) MM.1S cells overexpressing Mcl-1 protein (MM.1S/Mcl-1) were homogenized in 2% CHAPS-containing buffer, immunoprecipitated with anti-Bim antibody and analyzed by western blot with anti-Mcl-1 and anti-Bim antibodies as in b As shown in the left panel, MM.1S/Mcl-1 cells overexpress an alternative splicing Mcl-1 isoform [39], marked with an *, which retains the ability to bind Bim. In all cases, results from a single representative experiment, out of five, is shown.

gene-silencing experiments further supported the main role of Bim in this apoptosis (see later).

According to previous reports [12], the apoptotic ability of Dex was greatly potentiated by cotreatment with rapamycin. Growth inhibition caused by rapamycin alone in either Dex-sensitive or resistant MM cell lines was mainly due to cell cycle arrest. We have found that co-treatment with rapamycin greatly increased $\Delta\Psi_m$ loss, caspase-3 activation PS exposure and apoptosis in Dex-sensitive cells. In a previous work, in an attempt to identify the molecular determinants of rapamycin sensitization, the translational state of apoptosis-regulating proteins was analyzed. By using a high-throughput microarray methodology [13], an increase in mRNA translational efficiency for FasL, Bim and p53, and a decrease in that of XIAP was observed in OPM-2 cells treated with

rapamycin. Western blot analysis confirmed the rapamycin-induced decrease of XIAP protein levels but no increases of levels or activities of proapoptotic proteins were detected [13]. In this regard, we have found that MM.1S and OPM-2 cells express FasL in cytoplasm but not in plasma membrane, and that levels of this protein did not change after Dex or rapamycin treatment. The possible participation of Apo2L/TRAIL can also be ruled out since we previously reported that rapamycin does not affect to TRAIL-induced apoptosis [27] while increasing Bim protein levels [14] in MM cells. We also confirm the reduction in XIAP levels in OPM-2 cells upon treatment with rapamycin and we have also found a decrease in mRNA and protein levels of survivin, another IAP protein. More important, we have found that the BH3-only protein Bim is the key mediator of Dex-induced apoptosis and of its potentiation by rapamycin. By using a sensitive RT-MLPA analysis, and in agreement with the previous work [13], we have found that Bim mRNA levels increase in MM cells treated with Dex alone or in combination with rapamycin. Western blot analysis show a moderate increase in Bim protein levels in those cells. The importance of Bim as critical mediator of apoptosis induced by Dex and responsible for the potentiating effect of rapamycin was also supported by co-immunoprecipitation and gene-silencing

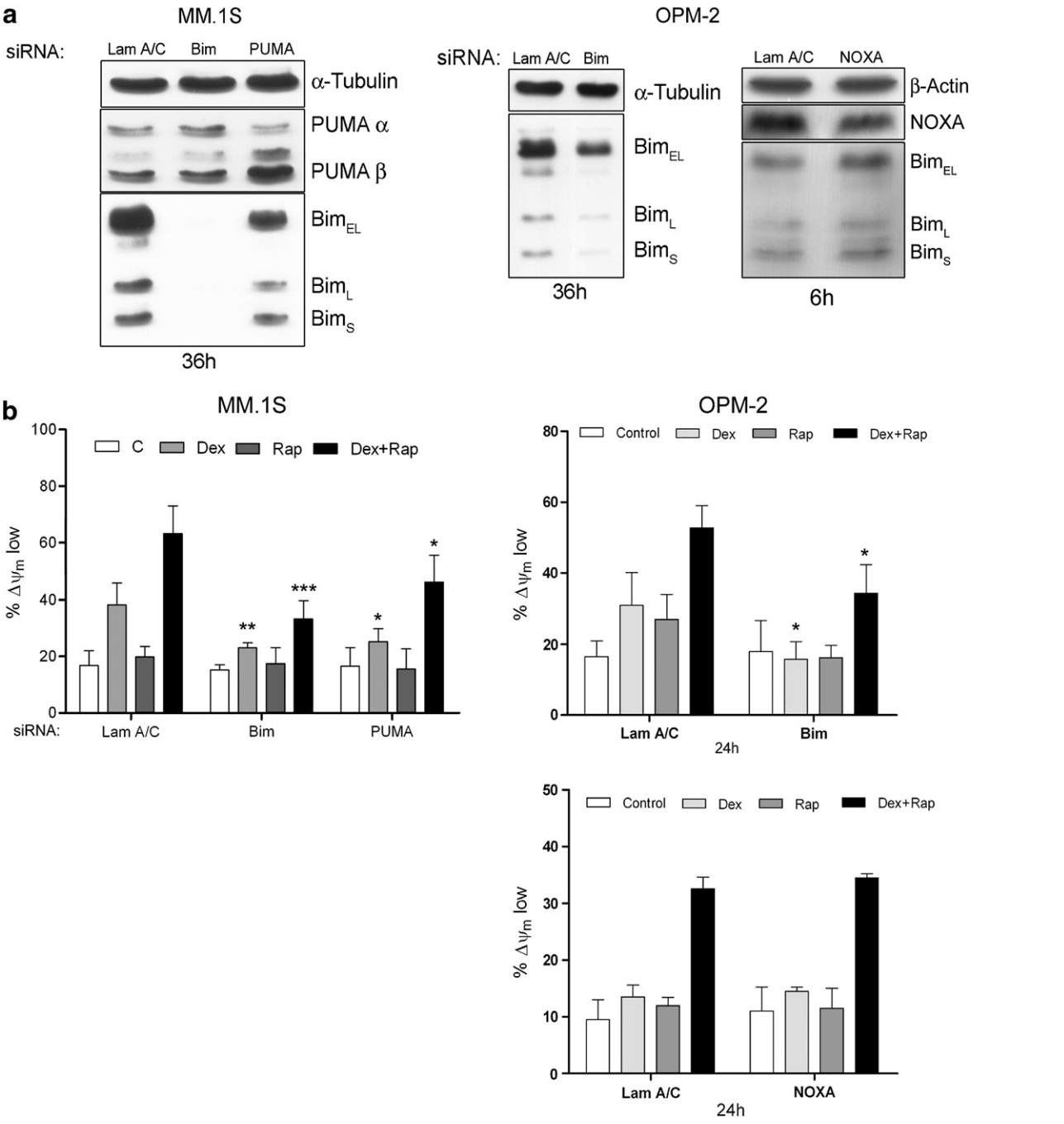

Fig. 7. Effect of gene silencing of Bim or Puma on Dex-induced apoptosis in MM.1S cells. (a) MM.1S and OPM-2 cells were transfected with the siRNAs for Lamin A/C (control), Bim, Puma or Noxa, as indicated, with a Nucleofector (MM.1S) or with HiPerFect reagent (OPM-2). Transfected cells were cultured for the times indicated and protein levels analyzed by Western blotting to verify protein silencing. (b) Cells were then treated with either 5 μM Dex, 10 nM rapamycin or both, for 24 h, and apoptosis determined by measuring $\Delta\Psi_m$ with TMRE by flow cytometry. (c) Western blot analysis of Bim (MM.1S) or Bim and Noxa (OPM-2) proteins in cells treated for 24 h with Dex and/or rapamycin. A representative blot, out of three, is shown.

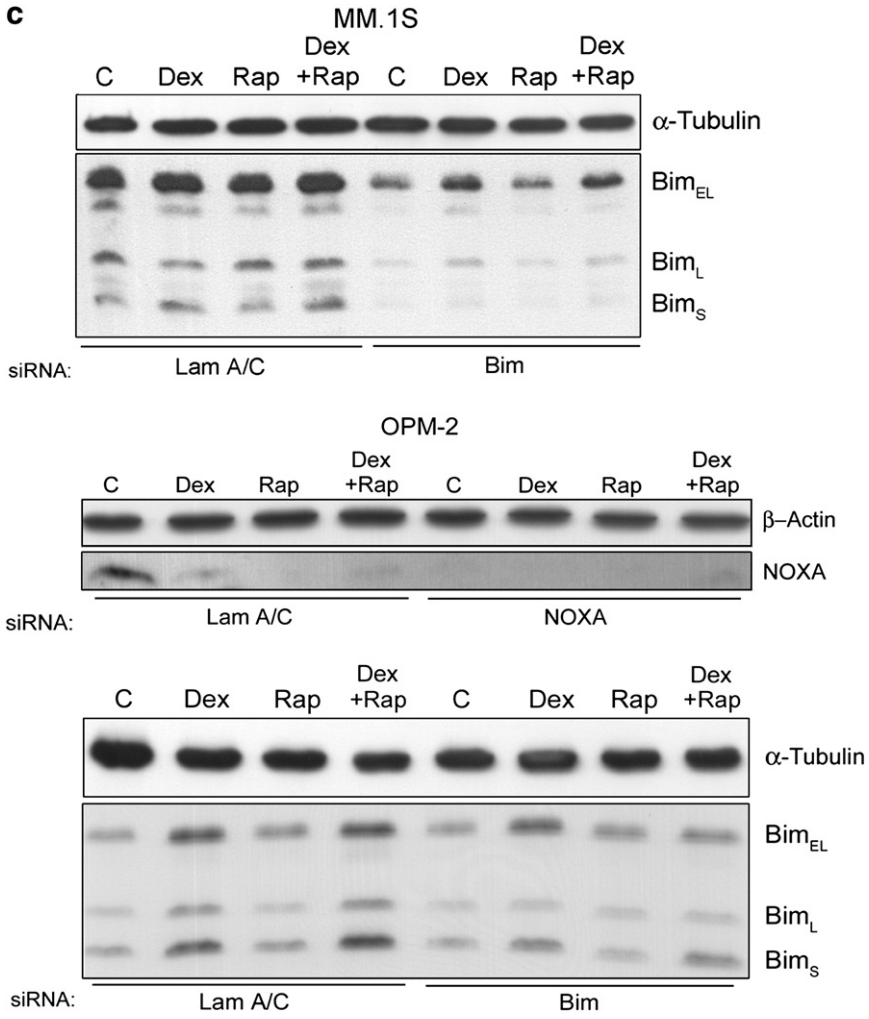

Fig. 7 (*continued*).

experiments. It was previously reported that Mcl-1 binds to Bim and behaves as a natural antagonist of Bim proapoptotic function [22,28]. In MM cells, Dex treatment, and in a greater extent Dex + rapamycin, induced the release of Bim from Mcl-1 and its association with a CHAPS-insoluble fraction containing Bak and membrane mitochondrial markers. This accumulation of Bim in the "mitochondria-rich," insoluble cell fraction, upon apoptosis induction has been previously reported by others [29]. To further clarify the respective role of Bim and Mcl-1 in Dex-induced apoptosis, we also analyzed the interaction of Bim and Mcl-1 in MM cells overexpressing Mcl-1. When these cells were treated with Dex or Dex + rapamycin, Bim remained associated to Mcl-1, it was not found in the CHAPS-insoluble fraction and apoptosis was blocked. On the other hand, it has been reported that, once released from Mcl-1, Bim can activate Bax and/or Bak [30,31]. Direct association between Bax or Bak with Bim in model systems has also been described [30,32]. However, direct association of Bim or other BH3-only proteins in cells under physiological conditions has not convincingly demonstrated [33], suggesting that any putative direct binding between Bim and Bax/Bak is presumably short-lived. Our present results agree with this hypothesis and with results from previous studies [29]. Also according to these results, transfection with Bim siRNA abrogated Dex-induced apoptosis in MM.1S and OPM-2 cells and also most of the potentiating effect of rapamycin. The slight protective effect of Puma-siRNA2 in MM.1S cells treated with Dex or Dex + rapamycin was associated with a slight decrease in Bim levels, perhaps due to an off-target effect. Anyway, a small contribution of Puma to apoptosis cannot be ruled out. Alternatively, and since Bim silencing did not fully protect from apoptosis induced by Dex + rapamycin another uncharacterized BH3-only, Mcl-1-interacting protein can be responsible for the potentiating effect of rapamycin. In this connection, IM-9 cells, a B-leukemia considered to be for many years a myeloma, express functional glucocorticoid receptors (GR) but are insensitive to the apoptotic action of Dex [34,35]. These facts can be explained by our current results, since IM-9 cells express very low levels of Bim mRNA that are not increased by Dex treatment (Fig. S2), and do not express Bim protein [17].

A very recent report has investigated the mechanism of Dex-induced apoptosis in MM.1S cells by using MM.1R-resistant cells transfected with different glucocorticoid receptor (GR) mutants [36]. According to our present results, re-expression of wild-type GR in MM.1R cells induced Bim expression and apoptosis. However, cells transfected with a mutant GR that neither binds to DNA nor induces transactivation [37], still up-regulated Bim upon Dex treatment but did not undergo apoptosis. This is a rather puzzling result but a possible explanation would be that those particular clones would express higher amounts of Mcl-1 than parental cells. Last, but not least, our results agree with the finding that B-lymphocytes from Bim-deficient mice are protected from dexamethasone-induced apoptosis [38].

In summary, our results indicate that the BH3-only protein Bim is the key mediator of apoptosis induced in myeloma cells by Dex and also of its potentiation by the mTOR inhibitor rapamycin. Our results also suggest that Mcl-1 is the main anti-apoptotic protein charged of neutralizing Bim action. Last, but not least, these data provide

molecular criteria for the use of Dex alone or combined with rapamycin in myeloma therapy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamcr.2009.11.004.

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